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13. ABSTRACT (Maximum 200 Words)

In this project, we proposed to use a dicistronic expression system to determine whether the long 5′-UTR sequence of PTEN contains internal ribosome entry site (IRES) which can mediate cap-independent translation. In the first year of the study, we have accomplished the proposed work as planed. We found that the long 5′-UTR sequence of PTEN inhibits cap-dependent translation and that a region in the 5′-UTR sequence of PTEN has an activity to enhance the expression of the second cistron in a dicistronic assay. However, during the second year of studies when the 5′-UTR sequence of PTEN was cloned into the promoterless dicistronic vector and tested for its stimulatory activity for the expression of the second cistron, we found that the 5′-UTR of PTEN has a strong promoter instead of the previously proposed IRES. We have now mapped this promoter and it is likely responsible for constitutive production of the PTEN mRNAs with shorter 5′-UTRs which would be compatible for cap-dependent translation initiation.

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INTRODUCTION

PTEN/MMAC1/TEP1 (referred as PTEN in the remaining text of this paper) is a tumor suppressor gene that maps to the 10q23.3 and has been shown to be deleted or mutated in many human tumors including glioblastomas, endometrial neoplasms, hematological malignancies, and prostate and breast cancers (Ali et al., 1999; Cantley and Neel, 1999; Dahia, 2000; Di Cristofano and Pandolfi, 2000). In addition, germline mutations in PTEN cause Cowden syndrome (CS), characterized by multiple hamartomas and a high proclivity for developing cancer (Cantley and Neel, 1999). The 5'-UTRs of human and mouse PTEN are highly homologous (the 0.9 kb 5'-UTR near the translation start codon shows ~90% homology) and have many features of a translationally-regulated gene. Firstly, they are significantly longer (>0.95 kb) than the average eukaryotic 5'-UTR. Secondly, they contain multiple short open reading frames (>3 for human and mouse PTEN). Thirdly, they are GC rich with predicted stable complex secondary structures. These features of 5'-UTR are expected to significantly hinder translation initiated by the conventional cap-dependent mechanism. Thus, an alternative mechanism such as internal ribosome entry may be involved in regulating the translation of PTEN. It is possible that in some advanced prostate cancers PTEN expression is omitted due to miss-regulation of IRES-mediated translation initiation. In this project, we plan to test this theory which is novel and has not been anticipated previously.

BODY

In the original application, we proposed to accomplish two tasks in the first year: (1) cap-dependent translation and IRES activity of PTEN/MMAC1 5'-UTR and (2) boundary of PTEN/MMAC1 IRES and mutagenesis effect. If it were true that the 5'-UTR of PTEN has an IRES, we would determine the sporadic mutations of PTEN/MMAC1 5'-UTR in advanced prostate tumor in the second year as task 3.

It has been argued that the use of dicistronic vector for determination of IRES activity suffers an inevitable drawback that the 5'-UTR sequence may contain a cryptic promoter (Kozak, 2001; Schneider and Kozak, 2001). In the past, this argument has been essentially ignored. During the first year of study, we demonstrated that the later case is true by using a promoterless dicistronic vector to test the well-known IRES of eIF4G (Gan et al., 1998; Gan and Rhoads, 1996). Although we have shown that the 5'-UTR sequence of PTEN potentially has a high IRES activity also during the first year of study, we needed to show that this IRES activity is not due to existence of a promoter. Thus, we had to postpone the original proposed task 3 and investigated instead if the 5'-UTR sequence of PTEN has any promoter activity. This study has now been published in Oncogene (22:5325-5337) (see appendix).

Using the promoterless dicistronic vector, we found that the 5'-UTR sequence of mouse PTEN does not have internal ribosome entry site (IRES) that can mediate cap-independent initiation of translation (see Fig. 6 in the appended reprint). Instead, we found that the 5'-UTR sequence of mouse PTEN contains a strong promoter that drives production of a transcript with shorter 5'-UTRs which can be translated with higher efficiency (see Fig. 2 and Fig. 7 in the appended reprint). We mapped this promoter to the region between –550 and –220 bases upstream of the translation start codon (see Fig. 8 in the appended reprint). Co-transfection analysis using *Drosophila* SL2 cells showed that Sp1 is one of the major transcription factors that can constitutively activate this promoter (see Fig. 9 in the appended publication). Two endogenous PTEN transcripts with 5'-UTRs of 193 and 109 bases were found in prostate cancer cell line DU145 (see Fig. 10 in the appended publication). Based on these observations, we concluded that the PTEN expression may be regulated at both transcriptional and translational levels and that the 5'-UTR sequence of PTEN contains a promoter that is responsible for constitutive PTEN expression.

Based on our findings on PTEN 5'-UTR sequence, we thought that the past studies on many cellular IRES elements may contain major flaws. To test this possibility, we took the well known cellular IRES derived from PDGF-B 5'-UTR and examined its IRES activity using our promoterless dicistronic vector. Similar to the findings of PTEN, the previously thought IRES of PDGF-B is also a promoter that drives the production of shorter transcripts which can be efficiently translated (see Fig. 1).

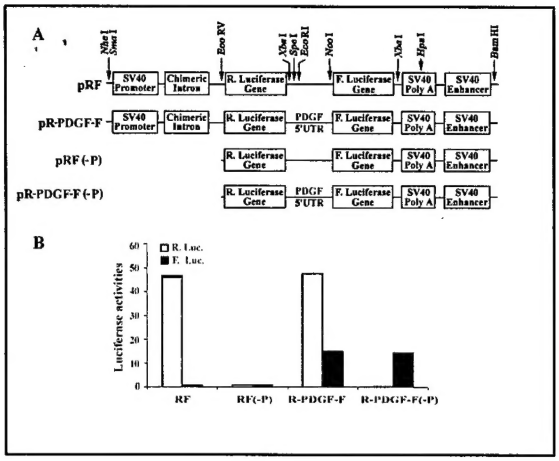

Figure 1. Dicistronic DNA test of the 5'-UTR sequence of human PDGF-B. (A) Schematic diagram of traditional and promoterless dicistronic constructs. The 5'-UTR sequence of human PDGF-B and the IRES element of HRV are cloned into the intergenic region of the dicistronic vectors. The locations of several relevant restriction enzyme sites are shown by arrows. In the promoterless constructs, the SV40 promoter and the chimeric intron sequence were deleted. (B) Relative luciferase activities generated by the dicistronic constructs in H1299 cells. H1299 cells were transfected with pRF, pR-HRV-F, and pR-PDGF-F constructs together with ß-Gal plasmid. Twenty-four hours following transfection, cells were harvested and the *Renilla* and firefly luciferases activities were measured and normalized against ß-galactosidase activity followed by normalization against the firefly luciferase activity of pRF. The data shown represent one of the four independent experiments. Clearly the firefly luciferase activity generated by the pR-PDGF-F construct is not due to its IRES but due to the promoter activity as shown by the promoterless construct pR-PDGF-F(-P).

We also examined another known cellular IRES derived from the 5'-UTR of p27 using our promoterless dicistronic vector. As shown in Fig. 2, we also found that the previously thought IRES of p27 is likely a promoter.

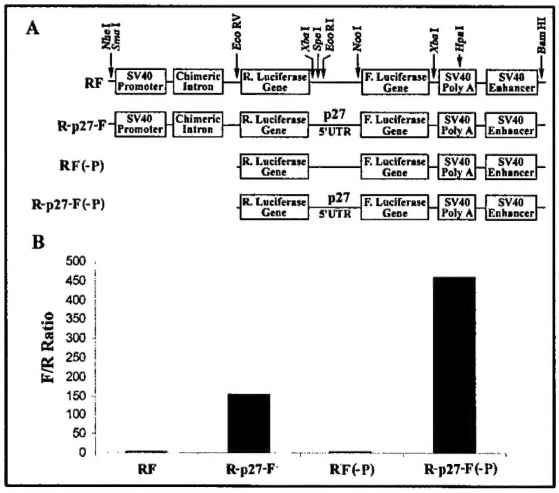

Figure 2. Dicistronic DNA test of the 5'-UTR sequence of human p27. (A) Schematic diagram of traditional and promoterless dicistronic constructs. The 5'-UTR sequence of human p27 was cloned into the intergenic region of the dicistronic vectors. The locations of several relevant restriction enzyme sites are shown by arrows. In the promoterless constructs, the SV40 promoter and the chimeric intron sequence were deleted. (B) Relative luciferase activities generated by the dicistronic constructs in HeLa cells. HeLa cells were transfected with pRF, and pR-PDGF-F constructs together with ß-Gal plasmid. Twenty-four hours following transfection, cells were harvested and the *Renilla* and firefly luciferases activities were measured and normalized against ß-galactosidase activity followed by normalization against the *Renilla* luciferase activity.

KEY RESEARCH ACCOMPLISHMENTS

1. Cloned the 5'-UTR of PTEN into the promoterless dicistronic assay system for IRES assay and found that the 5'-UTR of PTEN does not have an IRES as previously thought and it contains cryptic promoters which is responsible for constitutive PTEN expression.
2. The promoter in the 5'-UTR of PTEN has been mapped to the region between -550 and -220 bases upstream of the translation start codon.
3. Sp1 transcription factor was found to be the major factor responsible for internal transcription initiation.

REPORTABLE OUTCOMES

1. Han, B,; Dong, Z.; Liu, Y.; Chen, Q.; Hashimoto, K.; **Zhang, J.T.** Regulation of constitutive expression of mouse PTEN by the 5'-untranslated region. *Oncogene* 22:5325-5337; 2003.

CONCLUSIONS

In conclusion, the long 5'-UTR sequence of PTEN contains promoters that mediate production of PTEN mRNAs with shorter 5'-UTRs which are likely responsible for constitutive production of PTEN protein. The mRNAs of PTEN derived from these promoters have shorter 5'-UTRs which are compatible with the 5'-cap

dependent translation initiation and ribosome scanning mechanism. The long 5'-UTR of PTEN likely does not have an IRES element as we proposed originally. Furthermore, the past studies on many cellular IRES elements may contain major flaws and a new test using promterless dicistronic vector needs to be used to safeguard the claim of cellular IRES.

In the initial review, based on our new findings it was recommended that a revised statement of work needs to be submitted. In light of our findings that the 5'-UTR of PTEN does not have a predicted IRES but rather promoters, we revised our goals for the third year as following:

Months 25-31: To investigate the 5'-UTR promoter activity of PTEN constructs in prostate cancer cell lines in comparison with normal prostate epithelial cells using transient transfection.

Months 32-36: To determine the expression level of PTEN transcripts with shorter 5'-UTRs in prostate tumors in comparison with adjacent normal prostate epithelial cells using RNase protection assay.

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Regulation of constitutive expression of mouse PTEN by the 5′-untranslated region

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PTEN tumor suppressor serves as a major negative regulator of survival signaling mediated by PI3 kinase/AKT/protein kinase B pathway, and is inactivated in various human tumors. Elucidation of mechanisms responsible for PTEN expression is important for providing insight into strategies to control the loss of PTEN expression in human cancers. Although recent studies suggested that p53 and Egr-1 can modulate induced PTEN expression, the mechanism responsible for ubiquitous constitutive expression of PTEN remains elusive. PTEN mRNA contains a highly conserved and GC-rich 5′-untranslated region (5′-UTR). Recently, it has been shown that the long 5′-UTR sequences of several growth-regulated mRNAs contain promoters that can generate mRNAs with shorter 5′-UTRs. In this paper, we tested whether the 5′-UTR sequence of mouse PTEN contains a promoter that is responsible for constitutive expression of PTEN. We found that the long 5′-UTR sequence of mouse PTEN severely inhibits translation of PTEN and a heterologous gene firefly luciferase. Deletion of the most 5′-UTR sequence would enhance translation efficiency 100-fold. We also showed that the 5′-UTR sequence of mouse PTEN does not have an internal ribosome entry site (IRES) that can mediate cap-independent initiation of translation. Instead, we found that the 5′-UTR sequence of mouse PTEN contains a strong promoter that drives the production of a transcript with shorter 5′-UTRs, which can be translated with higher efficiency. This promoter was mapped to the region between −551 and −220 bases upstream of the translation start codon. Cotransfection analysis using *Drosophila* SL2 cells showed that Sp1 is one of the major transcription factors that can constitutively activate this promoter. Two endogenous PTEN transcripts with 5′-UTRs of 193 and 109 bases were found in DU145 and H226 cell lines. Based on these observations, we conclude that the PTEN expression may be regulated at both transcriptional and translational levels, and that the 5′-UTR sequence of PTEN contains a promoter that is responsible for constitutive PTEN expression.
Oncogene (2003) **22,** 5325–5337. doi:10.1038/sj.onc.1206783

Keywords: PTEN; IRES; promoter; 5′-UTR

Introduction

PTEN/MMAC1/TEP1 (referred as PTEN in the remaining text of this paper) is a tumor suppressor gene that maps to the 10q23.3 and has been shown to be deleted or mutated in many human tumors including glioblastomas, endometrial neoplasms, hematological malignancies, and prostate and breast cancers (Ali *et al.*, 1999; Cantley and Neel, 1999; Dahia, 2000; Di Cristofano and Pandolfi, 2000). In addition, germline mutations in PTEN cause Cowden syndrome (CS), characterized by multiple hamartomas and a high proclivity for developing cancer (Cantley and Neel, 1999). Although PTEN sequence is highly homologous with dual-specificity protein phosphatase, its major substrate is phosphatidylinositol triphosphate (PIP_3). It has been suggested that PTEN is a major negative regulator of PIP_3 and negatively regulates the survival signaling mediated by the PI3 kinase/AKT/protein kinase B pathway (Maehama and Dixon, 1999; Leslie and Downes, 2002). Loss of PTEN function or expression results in an increased concentration of PIP_3 and AKT hyperactivation, which leads to the protection of cells from various apoptotic stimuli (Stambolic *et al.*, 1998). In contrast, overproduction of PTEN induces growth suppression via cell cycle arrest and/or induction of apoptosis, and inhibits cell adhesion and migration (Dahia, 2000). Recently, PTEN has been demonstrated to serve as a negative regulator for proliferation of human neural stem cells and mammary epithelial cells (Backman *et al.*, 2001; Groszer *et al.*, 2001; Kwon *et al.*, 2001; Li *et al.*, 2002).

With the critical role in antagonizing PI3 kinase pathways, PTEN is anticipated to be the target of complex control mechanisms (Leslie and Downes, 2002). However, very little is known about the regulation of PTEN expression. PTEN expression is ubiquitous and constitutive, while it can also be altered by

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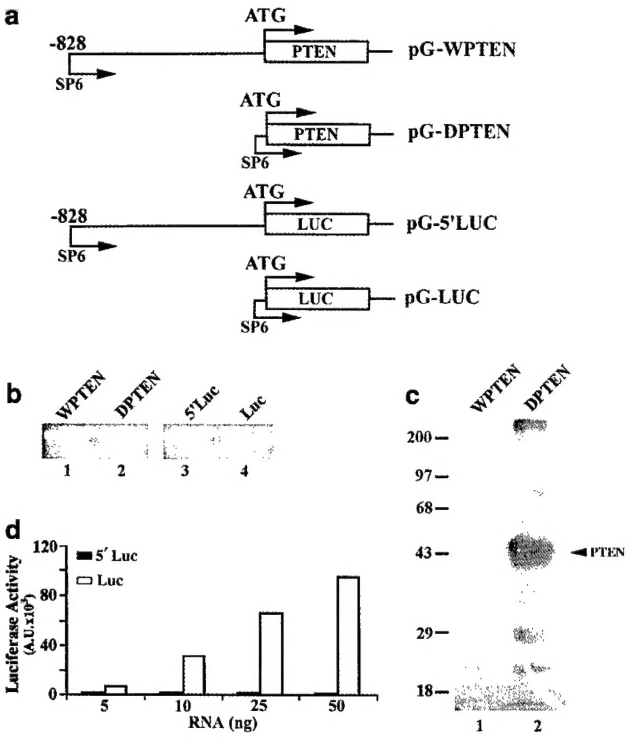

Figure 1 5′-UTR of PTEN inhibits translation of its downstream sequence in RRL. (**a**) Schematic diagram of *in vitro* transcription constructs. The transcription start site using SP6 RNA polymerase and the translation start codon AUG are indicated by arrows. (**b**) *In vitro* transcription. The capped RNA transcripts were generated by *in vitro* transcription using linearized plasmids and SP6 RNA polymerase, and 500 ng of each RNA transcript was separated using 1% agarose gel. (**c**) *In vitro* translation of mouse PTEN. The *in vitro* transcripts of mouse PTEN with (WPTEN) or without (DPTEN) the 5′-UTR sequence were used to program translation in RRL in the presence of [^{35}S]methionine. Protein product was separated by SDS–PAGE and visualized by autoradiography. (**d**) *In vitro* translation of firefly luciferase. 5, 10, 25, and 50 ng of *in vitro* transcripts of firefly luciferase with (5′Luc, solid bars) or without (Luc, open bars) the 5′-UTR sequence of mouse PTEN were used to program translation in RRL, followed by measuring luciferase activity by enzymatic assays

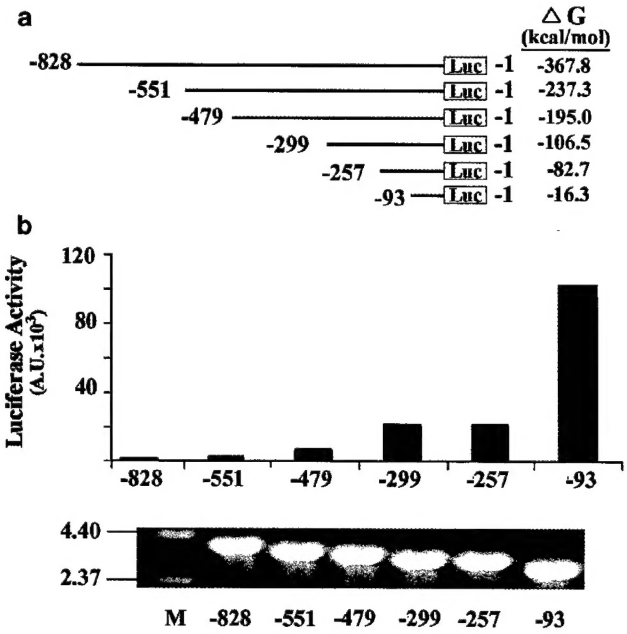

Figure 2 Effect of deletions of the 5′-UTR sequence of mouse PTEN on the translation of firefly luciferase in RRL. (**a**) Schematic diagram of sequential deletions in the 5′-UTR sequence of mouse PTEN. The free energy for the predicted secondary structure of the 5′-UTR sequence is shown on the right. The positions of the 5′ end of each deletion are indicated on the left. These mutant 5′-UTR sequences were engineered into pG-LUC for *in vitro* expression. (**b**) *In vitro* transcription and translation. The deletion mutants were used to generate *in vitro* transcripts (bottom), which were then used to program cell-free translation in RRL. LUC activities of the translated products were measured by enzymatic assays as described in Materials and methods

generated a significantly increasing amount of firefly LUC activity proportional to the amount of transcripts used, while no LUC activity was detected from the translation programmed by pG-5′LUC transcripts. Thus, the 5′-UTR sequence of mouse PTEN inhibits translation of transcripts encoding a heterologous protein and is independent of the protein-encoding sequence.

To further analyse the translational inhibition effect of the 5′-UTR sequence of PTEN, we generated several sequential deletion mutants from the 5′ end of the 5′-UTR in pG-5′LUC constructs (Figure 2a). These deletion mutants contain putative secondary structures with different free energies from −367 to −16 kcal/mol predicted by Zuker's mfold program (Mathews *et al.*, 1999). As shown in Figure 2b, translation of firefly LUC was very inefficient for transcripts with 5′-UTRs that have 479 nucleotides or longer. Further deletion resulted in an increase in translation. However, significant translation occurred only to the transcript with 93 bases of the 5′-UTR sequence with a predicted energy of −16 kcal/mol. It is ~100-fold more translatable than the full-length transcript. These results are consistent with a model that the stable secondary structure in the 5′-UTR sequence of PTEN mRNA inhibits the ribosome scanning process for cap-dependent translation initiation.

5′-UTR sequence of PTEN enhances expression of the second cistron in dicistronic test

The above results clearly demonstrate that the 5′-UTR of PTEN mRNA can effectively suppress ribosome scanning *in vitro*. However, PTEN protein has been reported to be constitutively expressed and its expression can be induced to a high level by biological stimulation. Thus, if PTEN mRNA with the full-length 5′-UTR is translated, it may use an alternative mechanism such as IRES-mediated translation initiation. To test this hypothesis, the 5′-UTR (−828 to −1 bases) of PTEN was cloned into the intergenic region of a dicistronic vector pRF (Stoneley *et al.*, 2000) to obtain pR-PTEN-F (Figure 3a). The internal ribosome entry site (IRES) sequence of human rhinovirus (HRV) was engineered in the same way and was used as a positive control. The pRF-based constructs contain an SV40 promoter to direct the transcription of dicistronic RNA encoding *Renilla* LUC as the first cistron and firefly LUC as the second cistron. Translation of the first

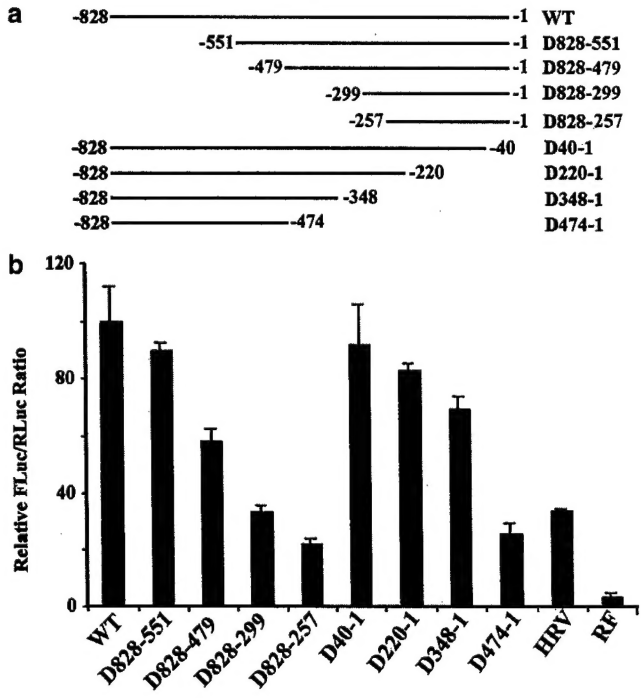

Figure 4 Deletion mapping of the 5′-UTR sequence of mouse PTEN in dicistronic DNA test. (**a**) Schematic diagram of sequential deletions of the 5′-UTR sequence of mouse PTEN. The positions of the 5′ and 3′ ends of each deletion are indicated on the left and right, respectively. These mutant 5′-UTR sequences were engineered into the dicistronic vector pRF at the intergenic region. (**b**) Relative luciferase activities generated from constructs containing the wild-type and mutant 5′-UTR sequences of mouse PTEN. HeLa cells were transfected with the constructs shown in (**a**), and 24 h following transfection, *Renilla* and firefly LUC activities were measured and the ratio of firefly to *Renilla* LUC was calculated and normalized to the wild-type (WT) control. The empty vector pRF and the plasmid containing HRV IRES were also used as negative and positive controls, respectively. The data were from three independent experiments

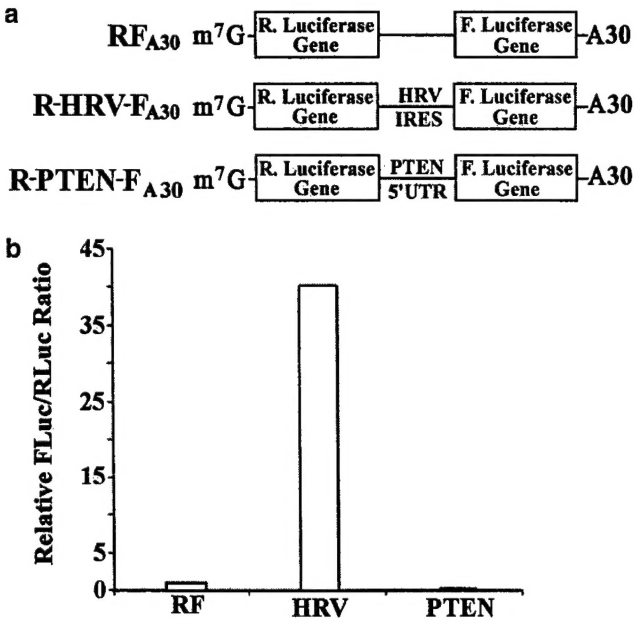

Figure 5 Translation of dicistronic mRNA in HeLa cells. (**a**) Schematic diagram of the dicistronic mRNA used for translation in HeLa cells. *In vitro* transcripts with 5′ cap (m^7G) and 3′ poly A tail (A_{30}) were synthesized using T7 RNA polymerase from linearized vector (RF_{A30}) and constructs containing the IRES of HRV (R-HRV-F_{A30}) and the 5′-UTR of PTEN (−551 to −1) (R-PTEN-F_{A30}). (**b**) Relative LUC activity from dicistronic mRNAs in HeLa cells. HeLa cells were transfected with the dicistronic mRNAs, and 8 h following transfection, *Renilla* and firefly LUC activities were measured and the relative ratios were calculated and normalized to that of the vector-transfected cells (RF_{A30})

encodes the −492 to −1 region of the 5′-UTR sequence of human PTEN enhanced the expression of the second cistron by 50-fold (data not shown), equivalent to that of the D828-479 construct of mouse PTEN (Figure 4b). Similar results with these constructs have also been observed by using another cell line H1299 (data not shown). Since the deletion mutant D828-551 and D220-1 retains the most enhancing activity, the 230-nucleotide central region of the 5′-UTR (−551 to −220) may contain important elements for stimulating firefly LUC expression. It is noteworthy that deletion mutants that retain about 200 nucleotides of the 5′-UTR sequence at the 3′-end (D828-257) or at the 5′-end (D474-1) still have the enhancing activity comparable to HRV IRES, suggesting that the enhancing activity also exists at both 5′- and 3′-ends of the 5′-UTR sequence of PTEN.

5′-UTR of PTEN does not display an IRES activity in dicistronic mRNA assay

The above results suggest that the 5′-UTR of PTEN may (1) contain an IRES activity that enhances the translation of firefly LUC from the dicistronic mRNA by internal initiation (for reviews see Pestova *et al.* (2001) and Sachs (2000)), (2) contain a promoter that directs transcription of the firefly LUC gene (Han and Zhang, 2002), and/or (3) contain a splicing acceptor site, that creates a splicing variant with only the second cistron of the firefly LUC gene. To distinguish between these possibilities, we generated dicistronic RNAs *in vitro* from the dicistronic constructs and used them to program translation both in HeLa cells and in RRL. RNA transfection allows a direct analysis of whether the 5′-UTR sequence of PTEN in the intergenic region of a dicistronic mRNA can enhance the translation of the second cistron without transcriptional interference. For purposes of RNA transfection, plasmids pSP-RF_{A30}, pSP-R-PTEN-F_{A30}, and pSP-R-HRV-F_{A30} were engineered and used for producing dicistronic transcripts containing m^7GpppG cap and polyadenylated tail *in vitro* (Figure 5a). The *in vitro* transcripts were introduced into HeLa cells by lipofectin encapsulation. At 8 h following transfection, cell lysates were prepared for LUC activity measurement. As expected, the firefly LUC of vector RNA was very poorly translated and its activity (arbitrary units) only represented about 0.16% of *Renilla* LUC (data not shown). It increased to about 6.7% with the dicistronic RNAs containing HRV IRES. Therefore, HRV IRES significantly stimulated the translation of firefly LUC about 40-fold over vector control (Figure 5b). However, no stimulation of firefly LUC expression was observed with the 5′-UTR of

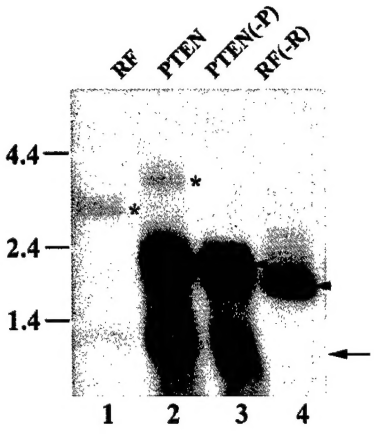

Figure 7 Northern blot analysis of RNA products generated by promoter of mouse PTEN. PolyA mRNAs were isolated following transfection with pRF (lane 1), pR-PTEN-F (lane 2), pR-PTEN-F(-P) (lane 3), and pRF(-R) (lane 4) and used for Northern blot analysis as described in Materials and methods. The asterisks and arrowheads indicate the dicistronic and monocistronic RNA transcripts, respectively. The arrow shows unknown RNAs hybridized with the probe which have been consistently observed in previous studies (Coldwell *et al*., 2000; Han and Zhang, 2002)

from pRF(-R) (lane 4), suggesting that these transcripts may be derived from multiple transcription start sites. Indeed, RT–PCR analysis showed the existence of transcripts starting from a very upstream region of the 5′-UTR (data not shown).

Transactivation of the promoter in the 5′-UTR sequence of PTEN by Sp1

To identify the boundaries of the DNA region that are responsible for promoter activity, deletion mutants were generated from either 5′ or 3′ ends of the 5′-UTR of PTEN (Figure 8a). These deletion mutants were engineered into the promoterless dicistronic vector and then used to determine their ability to direct firefly LUC expression. As shown in Figure 8b, deletion from either end of the 5′-UTR resulted in a gradual decrease in stimulating firefly LUC expression. The full stimulation activity resides in the region of −551 to −220 since deletion mutants D828-551 and D220-1 still contain full-promoter activity. In addition, the deletion mutants containing only the 5′ (D474-1) or 3′ (D828-257) region of the 5′-UTR sequence also have significant promoter activity. Widespread promoter activity within the 5′-UTR sequence of PTEN may be responsible for multiple transcriptional initiation sites observed with Northern blots. These observations are consistent with the results obtained from conventional dicistronic DNA constructs (see Figure 4).

Analysis of the potential transcription factor binding sites in the −551 to −220 region of the 5′-UTR sequence using MatInspector (Quandt *et al*., 1995) showed several consensus sites for transcription factors such as Sp1, Ets-1, Egr-1, Ap-1, Ap-2, and Ap-4 (Figure 9a). To determine directly whether the above transcription factors could functionally modulate promoter activity in the 5′-UTR sequence of PTEN, *Drosophila* SL2 cells,

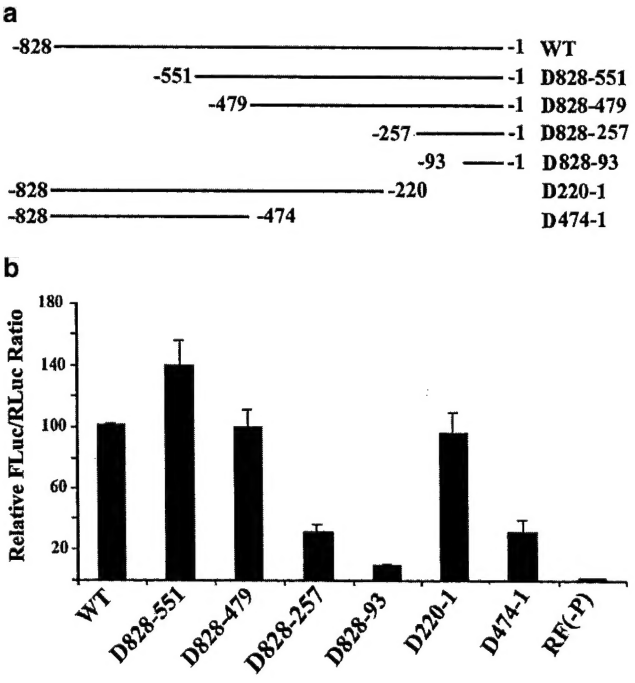

Figure 8 Deletion mapping of the promoter activity in the 5′-UTR sequence of mouse PTEN. (**a**) Schematic diagram of the deletions in the 5′-UTR of mouse PTEN. The positions of 5′ and 3′ ends of each deletion are indicated on the left and right, respectively. These mutant 5′-UTRs were engineered into the promoterless dicistronic vector pRF(-P) at the intergenic region. (**b**) Relative LUC activity generated from the wild-type and mutant 5′-UTR sequences of mouse PTEN. HeLa cells were transfected with the constructs shown in (**a**), and 24 h following transfection, *Renilla* and firefly LUC activities were measured and the ratio of firefly to *Renilla* LUC was calculated and normalized to the wild-type (WT) control. The data were from three independent experiments

which are deficient in Sp1-, Sp3-, and Ets-related proteins (Courey and Tjian, 1988; Dennig *et al*., 1996), were used. The reason for using insect instead of mammalian cells is that Sp1-, Sp3-, and Ets-related factors are expressed in virtually all mammalian cells, which could affect the interpretation of this experiment. We introduced the pR-PTEN-F(-P) construct along with *Drosophila* expression plasmids pPacSp1 or pPacUEts-1 into *Drosophila* SL2 cells. As shown in Figure 9b, pPacSp1 stimulated the promoter activity in the 5′-UTR sequence of PTEN by 15-fold, whereas it stimulated vector control only by threefold. Ets-1 did not significantly stimulate the promoter activity in the 5′-UTR sequence of PTEN. These results suggest that Sp1 is a possible transcription factor that regulates the constitutive expression of PTEN by acting at the promoter within the 5′-UTR of PTEN. Since there are several Sp1 binding sites in the 5′-UTR sequence, it remains to be determined which Sp1 element is responsible for the promoter activity.

Detection of endogenous transcripts originating from the 5′-UTR promoter in human cancer cell lines

Previous research with human PTEN using Northern blot detected heterogeneous transcripts of a variety of

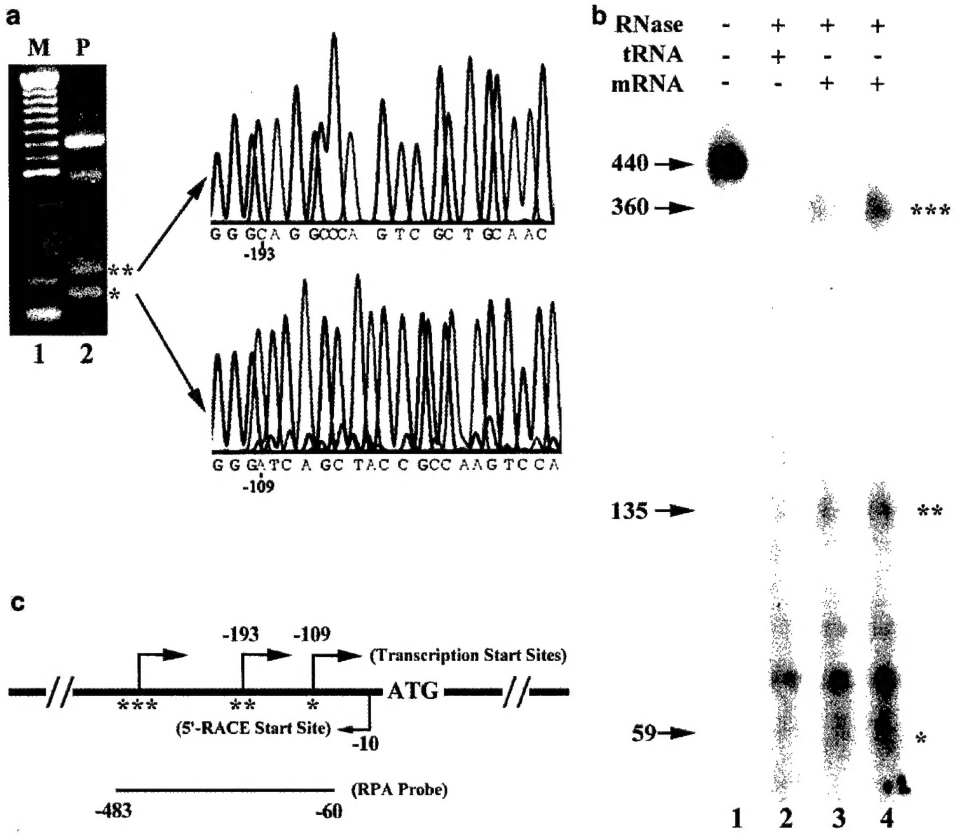

Figure 10 Endogenous PTEN transcripts with shorter 5′-UTRs exist in human cancer cell lines. (**a**) Mapping 5′-UTRs of endogenous PTEN transcripts by 5′ RACE. Total RNAs (2 μg) from DU145 cell line were used for 5′ RACE as described in Materials and methods. The final DNA products were separated by agarose gel electrophoresis (lane 2). The 5′ end sequencing results of the two small products were shown on the right. (**b**) Mapping 5′-UTRs of endogenous PTEN transcripts by an RNase protection assay. The RNase protection assay with 10 μg of RNAs from DU145 (lane 3) and H226 (lane 4) cells, 10 μg of yeast tRNA (lane 2), and a probe covering the region of −483 and −60 in the 5′ UTR sequence of human PTEN was performed as described in Materials and methods. The three distinctively protected products with estimated sizes of 360, 135, and 59 bases are indicated. Lane 1 shows the mobility and the estimated size of the probe used. (**c**) Schematic diagram showing relative locations of transcription start sites, the primer used in the 5′ RACE, and the probe used for RPA. The asterisks show the possible corresponding positions in the PTEN sequence for the products generated from both 5′ RACE (panel a) and RNase protection assay (panel b)

sequence in an *in vitro* system. A transcript with a 5′-UTR of only 93 bases is 100-fold more translatable than the transcript with a long 5′-UTR of 828 bases. The inhibitory effect of the long 5′-UTR is similar to that of the 5′-UTR of PDGF, which inhibits translation about 40-fold (Rao *et al*., 1988).

Inhibition of translation by 5′-UTR led us to explore other mechanisms such as IRES that may be used for PTEN translation. The 5′-UTR sequence of PTEN enhanced the expression of the second cistron by about 70-fold in the traditional dicistronic DNA assays and translation of the second cistron persisted when a hairpin structure was used to block translation of the first cistron (Figure 3). However, no such enhancing activity was observed in the dicistronic RNA assay, indicating that the 5′-UTR of PTEN may not have IRES. This conclusion is supported by our promoterless dicistronic test, which revealed that the enhancing effect was due to the existence of promoter activities in the 5′-UTR of PTEN. In addition, Northern blot analysis clearly detected the monocistronic transcript derived from the promoter in the 5′-UTR sequence of PTEN (Figure 7). By construction of a variety of deletion mutants from both 5′ and 3′ end of the 5′-UTR, we mapped the promoter activity to the region −551 to −220. Our experiments using *Drosophila* SL2 cells showed that the promoter in the 5′-UTR sequence of PTEN responds to Sp1, a ubiquitous transcription factor that is not only important for constitutive expression of many house-keeping genes but also critical for induced expression of many growth-related genes (Black *et al*., 2001). Finally, using the RNase protection assay and 5′ RACE, we identified two endogenous PTEN transcripts with short 5′-UTRs of 109 and 193 bases in both human cancer cell lines H226 and DU145, which were known to express human PTEN (Whang *et al*., 1998; Soria *et al*., 2002).

In the past, promoters in the 5′-UTR sequences have primarily been ignored and lack attention. Promoter studies normally begin with primer extension aiming at identification of the transcription start site furthest away from the translation initiation start codon. Subsequent analysis usually focuses on the promoter regions that are responsible for the generation of the transcript with the

be potential methylation targets as predicted using an online program (http://www.ebi.ac.uk/emboss) (data not shown). Indeed, the presence of methylation in the 5′-UTR sequences (−405 to −104) was reported to be frequent in both endometrial and gastric carcinomas (Salvesen *et al*., 2001; Kang *et al*., 2002). This region with high frequency of methylation overlaps with the region containing the promoter mapped in this study (−551 to −220). Methylation of this region may shut off 5′-UTR promoter activity and constitutive expression of PTEN and, therefore, inactivate the expression of PTEN in some tumors. We are currently testing this hypothesis by determining the effect of methylation of the 5′-UTR sequence on promoter activity.

Since the promoter in the 5′-UTR sequence of PTEN may serve as a major promoter controlling constitutive expression of PTEN, detailed promoter mapping may lead to discovery of *cis*-elements and transcription factors that govern constitutive PTEN expression. Analysis of putative transcription factor binding sites showed that the promoter in the 5′-UTR sequence contains several binding sites for transcription factors such as Sp1, Egr-1, Ets-1, Ap-1, Ap-2, and Ap-4. We have shown that Sp1 is involved in the activation of this promoter. Interestingly, the Sp1 site located at −82/−77 plays a critical role in the p53/p53 site (at 2281/2262)-mediated synergistic transactivation of the p21 promoter (Koutsodontis *et al*., 2001). Similarly, conditional formation of transcriptional Sp1–p53 regulatory complexes has been reported recently in a number of other promoters (Borellini and Glazer, 1993; Gualberto and Baldwin, 1995; Ohlsson *et al*., 1998; Torgeman *et al*., 2001). Sp1/p53 synergism may serve as a general mechanism for transcriptional activation of p53 target genes. Therefore, it is tempting to hypothesize that the possible synergism between the p53 site located in −1190 to −1157 of human PTEN promoter and the multiple Sp1 sites located in the 5′-UTR region also function on PTEN promoter. On the other hand, multiple consensus Egr-1 sites (GCGGCGGCG) identified in human PTEN promoter (Virolle *et al*., 2001) were also found in the 5′-UTR sequence of mouse PTEN (Figure 9a). Whether these Egr-1 sites are functional and whether the Sp1 and Egr-1 transcription factors interplay on the promoter in the 5′-UTR sequence of PTEN remain to be addressed in future studies.

Materials and methods

Materials

Restriction enzymes, m^{7}GpppG cap analogue, and Pfu polymerase were purchased from New England Biolabs, Amersham/Pharmacia Biotech, and Stratagene, respectively. The SL2 cell line was from ATCC. Sp6 RNA polymerases, RNasin, RNase-free DNase, RRL, LUC assay 'Stop & Glo' kit, and pSP64 PolyA plasmid were from Promega. RNeasy Mini Kit and Oligotex mRNA Mini Kit were from Qiagen. Rediprime II Random Prime Labeling System, α[^{32}P]dCTP and α[^{32}P]CTP were from Amersham Biosciences. The Sephadex G-25 Quick Spin Columns (TE) for radiolabeled DNA and RNA purification was from Roche Diagnostics. MAGNA nylon transfer membrane was from Osmonics Inc. Zero Blunt PCR Blunt PCR Cloning kit, Schneider's *Drosophila* culture medium, Lipofectamine plus and Lipofectin transfection reagent, and the 5′-RACE system for rapid amplification of cDNA ends were purchased from Invitrogen. Oligonucleotides were synthesized by Sigma-Genosys. IMAGE EST clones were obtained from either ATCC or Research Genetics. MAXIscript *in vitro* transcription kit and RPA III ribonuclease protection assay kit were products of Ambion. TaKaRa LA *Taq* polymerase with GC buffer was purchased from TaKaRa Bio Inc.

Construction of plasmids

The cDNA clone mncb-0146 (GeneBank Accession #: AU035162) was from the Sugano mouse brain mncb library (Suzuki *et al*., 2000). Double-strand DNA sequencing showed that this clone encodes the full-length mouse PTEN protein and confirmed that the 5′-UTR sequence (−828 to −1) was identical to that shown in GeneBank (Accession # *NM_008960*). The cDNA, following sequencing, was cloned into pGEM-4Z (Promega) at *Eco*RI and *Xba*I sites, resulting in the plasmid pG-WPTEN (Figure 1a). In the deletion construct pG-DPTEN, the 5′-UTR sequence from −828 to −8 was removed by replacing the *Eco*RI/*Bgl*II fragment representing −828 to +318 with a PCR fragment representing −8 to +318. The plasmid pG-LUC was generated by cloning *Eco*RI–*Xba*I fragment of pRF (Stoneley *et al*., 2000), corresponding to the firefly LUC-encoding region into pGEM-4Z. To generate pG-5′LUC, the −828 to −1 of the 5′-UTR sequence of mouse PTEN was amplified by PCR and inserted into pG-LUC between the *Eco*RI and *Nco*I sites. The 5′-UTR deletion mutants of mouse PTEN (−551/−1, −479/−1, −299/−1) were generated by cloning *Xho*I-*Nco*I, *Not*I-*Nco*I, *Sma*I-*Nco*I fragments into pG-LUC, respectively. The −257/−1 and −93/−1 deletion mutants were generated by PCR.

Dicistronic constructs were generated based on pRF. The 5′-UTR sequence of mouse PTEN (−828 to −1) from pG-LUC was cloned into pRF, resulting in pR-PTEN-F. In addition, a 492 bp of the 5′-UTR sequence of human PTEN cDNA (−492 to −1) was amplified from human IMAGE 2157760 (GeneBank Accession #: AI480306) and cloned into pRF, resulting in pR-hPTEN-F. Dicistronic constructs with a hairpin structure were generated by inserting into *Eco*RV site of pRF and pR-PTEN-F a double-stranded oligonucleotide with sense sequence of 5′-ATCAAAAGCGCAGGTCGCGACCGCG-CATGCGCGGTCGCGACCTGCGCTAAAGAT-3′. The dicistronic constructs containing the truncated 5′-UTR of PTEN (−551/−1, −479/−1, −299/−1, −257/−1) were generated by cloning these cDNA fragments from the pG-LUC-based constructs (see above) into pRF. The dicistronic constructs containing other truncations (−828/−220, −828/−348, and −828/−474) were obtained by deletions from pR-PTEN-F between *Bst*XI and *Nco*I, *Sma*I and *Nco*I, and between *Not*I and *Nco*I. The −828/−40 construct was generated by cloning a PCR fragment into pRF. The promoterless dicistronic constructs were generated as previously described (Han and Zhang, 2002).

Constructs containing poly(A) for *in vitro* transcription were engineered using the vector pSP64 PolyA (Promega) that has 30 bp (dA-dT) sequence. The *Eco*RV–*Xba*I fragment of the pRF vector containing the *Renilla* LUC gene was first cloned into pSP64 PolyA vector at the *Xba*I and blunted *Hind*III sites to generate the plasmid pSP-R_{A30}. The *Xba*I fragment of pR-HRV-F containing the IRES of HRV and the firefly LUC gene

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